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Abstract

While thymopentin has been used for many years in the experimental treatment of Sézary syndrome (SS), a rare and very aggressive lymphoma, its mechanism of action is still not known. Herein we show that this peptide acts as an inhibitor of isolated iNOS and nNOS isoforms, and reduces iNOS protein/mRNA levels and iNOS activity in blood cells obtained from both healthy donors and SS patients. Similar results were obtained with TPN-2, the N^{ω}-nitro analogue of the Arg-Lys motif present in thymopentin. Additional investigations are necessary to confirm the role and the relative importance of the two mechanisms of iNOS down-regulation in the therapeutic action of these peptides against SS.

Keywords

Nitric oxide synthase; Sézary syndrome; orphan disease; thymopentin.

1. Introduction

Orphan diseases are diseases so rare that the interest of pharmaceutical companies in developing drugs for their treatment is limited, because of the lack of profit potential. The medicines used to manage these diseases are called orphan drugs, and their development is largely in the hands of academicians. According to the National Institutes of Health Office of Rare Diseases, about 6800 such diseases are known to date [1,2]. Sézary syndrome (SS) is an example of orphan disease. SS is considered a rare and very aggressive leukemic variant of CTCL (cutaneous T cell lymphoma), characterized by extensive skin involvement (erythroderma), general lymphadenopathy, and by the presence of a malignant circulating T cell clone that over-expresses CD60 and lacks CD26, CD7, and CD49 [3]. CTCLs are the most frequent form of

extranodal non-Hodgkin lymphomas (NHL), accounting for 0.5-3% of all NHLs [4]. SS is an aggressive disease with a poor prognosis, median survival being 2 to 4 years [5]. The aetiology of the disease is still largely unknown. Except for a small number of therapeutic agents (interferons), there are no reliable efficacy data on agents used to treat SS patients [6]. A better understanding of the immunopathogenesis of this disease could aid in finding a more effective therapy. SS is associated with significant immune abnormalities characterized by dysregulation of both cellular and humoral immunity. In general, a shift in cytokine response from Th1 to Th2 is prominent in this disease, with concurrent down-regulation of IFN-γ and up-regulation of IL-10, IL-4 and IL-5. The latter are associated with defective neutrophil function and a reduction in cutaneous dendritic cells, as a result of the abnormal cytokine milieu [6]. Control of lymphocytic function and proliferation is of the utmost importance in the physiological balance of the immune system; alteration of this homeostasis may be important in the development of cancer or autoimmunity.

Nitric oxide (NO) is a pleiotropic messenger produced from L-arginine by the enzyme NO-synthase (NOS). This enzyme exists in three distinct isoforms, eNOS, nNOS and iNOS. The first two are constitutive isoforms, while the third is an inducible isoform. NO is involved in a variety of physiological and pathological processes; it is also one of the final effectors in the immune system, where it is generated in large amounts (mM range) after induction of iNOS, in particular by macrophages. It diffuses into parasitic cells or into cancer cells, and kills them by producing reactive species and by inhibiting enzymes or other functional proteins [7,8]. In tumours, NO regulates a number of signalling pathways, and its effects are complex and closely dependent on concentration. A large body of experimental evidence demonstrates that NOS levels, in particular the iNOS level, are up-regulated in tumours [9,10]. This observation has led

to strategies aimed at blocking experimental tumour growth by using iNOS inhibitors [11,12]. NO is one of the most important factors involved: it regulates both the apoptosis of potential cancer cells and the responsiveness of lymphocytes to a certain antigen, as well as T lymphocyte proliferation and dendritic cell apoptosis [13,14]. Moreover, lymphocytes themselves have the potential to generate NO through iNOS activation, which in turn modulates apoptosis and cytokine production [15]. There is experimental evidence on animal models that T cell-lymphoma tumour cells express large amounts of iNOS mRNA, and are characterized by high NO levels [16]. Conversely, it appears that iNOS may function as both an activator and an inhibitor of T cell apoptosis. In vitro, NO is capable of inducing apoptosis in cutaneous T cell lymphoma cells, by down-regulating NF-kB; therefore, it might act as a tumour suppressant [17]. In general, very little is known about the role of iNOS and NO in SS and in NHL. Genetic polymorphism in NOS genes, together with environmental factors such as diet, seems to modulate the risk of developing NHL in humans [18]. Moreover, other genetic variants of oxidative stress genes are also associated with an increased risk of developing NHL [19]. Thymopentin (TP-5), a synthetic pentapeptide containing residues 32-36 of the thymic hormone thymopoietin (Fig. 1), triggers in vitro and in vivo immunomodulatory effects, and consequently it might be a useful agent in treating SS [20,21]. Indeed, in a study of 20 patients with Sézary syndrome, Bernengo et al. observed that treatment with TP-5 produced eight complete and seven partial remissions [22]; these responses were achieved when circulating Sézary cells were below 2600 mm^{-3}. The mechanism of action of this peptide remains largely unknown.

This paper describes the results of a study carried out on TP-5, and shows this peptide to be an inhibitor of iNOS and, less effectively, of nNOS recombinant enzymes. In addition, we show that it is able to down-regulate the expression of iNOS mRNA/protein, as well as iNOS activity in the

peripheral blood adherent mononuclear cells (PBAMCs) of SS patients. The behaviour of some shorter peptides, TP-2, TP-3, TP-4, and of the analogues TPN-2, TPN-3, TPN-4, TPN-5, in which L-arginine is replaced by N^{ω}-nitro-L-arginine (Scheme 1), was also studied, for comparison purposes.

2. Materials and methods

2.1. Synthesis

N^{α}-Fmoc-protected amino acids, *O*-benzotriazole-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBt), 4-dimethylaminopyridine (DMAP) and Wang resin were purchased from GL Biochem (Shanghai, China). N^{α}-Boc-N^{ω}-nitro-L-arginine was purchased from Fluka (Buchs, Switzerland). All reagents and CH_3CN for HPLC were analytical grade and used without further purification. The peptides subject of this study were synthesised manually in a stepwise fashion via the solid-phase method [23], using a reaction vessel equipped with gas and solvent inlets/outlets; the contents were stirred by streaming a gentle nitrogen flux through the resin bed. HBTU and HOBt were used as coupling agents, in the presence of *N*,*N*-diisopropylethylamine (DIEA); the *C*-terminal amino acid was coupled to Wang resin (0.3 g, 1.3 mmol of NH_2 groups g^{-1}) under the same conditions, plus a catalytic amount of DMAP. The N^{α}-Fmoc protecting groups were removed by treating the protected resin-bound peptide with 25% piperidine in DMF (10 mL for 5 min, then 10 mL for 20 min). After washing with DMF (3×10 mL), synthesis proceeded to the next coupling step. A threefold excess of amino acid and coupling agents was used at each coupling step, to ensure completion of the reaction, which was routinely checked by the Kaiser test [24] on a few resin beads. Excess reagents were then washed with DMF (3×10 mL), CH_2Cl_2 (4×10 mL), and the

Fmoc deprotection protocol was repeated. After the last amino acid had been coupled, the *N*-terminal Fmoc group was removed as described above, then the peptide was released from the resin by treatment with TFA/Et_3SiH/H_2O (90:5:5) for 3 h. The resin was vacuum filtered, and the crude peptide was precipitated from the filtrate with cold anhydrous ethyl ether. All products were purified by RP-HPLC on a semi-preparative C18-bonded silica column (Vydac 218TP1010, 1.0×25 cm) using a gradient of CH_3CN in 0.1% aqueous TFA (from 10% to 50% in 45 min) at a flow rate of 1.0 mL min^{-1}. Title compounds were obtained as white solids by freeze-drying the appropriate fractions after removal of CH_3CN by rotary evaporation. Structures were confirmed by mass and NMR spectrometry, and purity of synthesized peptides was assessed as >99% by analytical RP-HPLC. Elemental analyses were performed by REDOX s.n.c. (Monza, Italy) and were within ±0.4% of the theoretical values. ^{1}H-NMR, ^{13}C-NMR and elemental compositions (C, H, N) are available as Supplementary material.

2.2. Cell sources and cultures

PBAMCs were obtained from ten healthy donors and from two Sèzary syndrome patients with circulating atypical T cells (CD4+ CD26-). The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans, and was approved by our ethical committee. All subjects gave their signed informed consent to participate in the study. PBAMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Isolation and purification of PBAMCs exploited their adherence to culture plates: after 1 h at 37 °C, non-adherent cells were removed with the supernatant, then washed three times with phosphate buffer solution. Adherent cells (more than 90% of which were CD14+) were carefully detached from the surface of the culture plates and re-suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 25 mM

Hepes buffer, 50 U mL^{-1} penicillin, and 50 mg mL^{-1} streptomycin; all media and components were purchased from SIGMA (Italy). Cells (10^6 mL^{-1}) were immediately plated and incubated at 37 °C in a cell culture incubator under a 95% humidified 5% CO_2 atmosphere.

2.3. Cell stimulation

The PBAMCs obtained from each subject, prepared following the same experimental procedure, were rinsed with fresh medium and stimulated with an inflammatory stimulant comprising LPS (10 μg mL^{-1}) and IFN-γ (100 U mL^{-1}), in the presence or in the absence of the test compound (100 μM). This concentration of the peptides was selected based on the results of pilot studies, using three different concentrations of the inhibitor (50, 100, 150 μM; data not shown). The test compounds were dissolved in sterile distilled water and added to cell cultures. An equal volume of medium was added to controls to normalize volumes. Prior to use, all reagents were tested for endotoxin (< 10 pg mL^{-1}; Associates of Cape Cod, Inc., Woods Hole, MA, USA) and mycoplasma contamination (General-probe II; General-probe Inc., San Diego, CA, USA) and found negative. The same batch of serum and of medium was used in all experiments. Cell viability in each culture was assessed by Trypan blue exclusion. Cytotoxicity experiments were performed using Cytotox 96 (Promega, Southampton, UK).

2.4. NOS activity

NOS activity was monitored using a hemoglobin capture assay [25]. A typical assay mixture for NOS contained 20 μM L-arginine, 100 μM NADPH, 10 μM tetrahydrobiopterin, 100 μM dithiothreitol, 1.6 mM $CaCl_2$, 11.6 mg mL^{-1} calmodulin, 3 mM oxyhemoglobin, plus the test concentration of inhibitor in 100 mM HEPES (pH 7.5). iNOS activity was assessed as described above, but in calcium-free conditions. All assays were carried out in a final volume of 1 mL and were initiated with enzyme. Nitric oxide reacts with oxyhemoglobin to yield methemoglobin,

which was detected at 401 nm (ε = 19,700 M^{-1} cm^{-1}) on a Perkin-Elmer LambdaBIO UV-vis spectrophotometer and recorded over 1 min. In addition, 50 units each of SOD and CAT were included in all assays, to minimize interference from reactive oxygen species, namely O_2^- and H_2O_2. All assays were performed at 37 °C and the results shown are the mean of three experiments. Cellular iNOS inhibitory activity was determined in the 11,000*g* supernatant of PBAMC homogenates. To ensure that the observed conversion of oxyhemoglobin to methemoglobin was amenable to NOS activity the hemoglobin capture assay was also repeated under the conditions described by Salter and Knowles [26]. Enzyme kinetic experiments were performed with recombinant enzymes. The NOS isoforms used were: iNOS (murine recombinant) expressed in E. coli, eNOS (bovine recombinant) isolated from a baculovirus overexpression system in Sf9 cells, and nNOS (rat recombinant) isolated from the baculovirus expression system in Sf21 cells.

The corresponding K_i values of TP-5 and TPN-2 inhibitors were calculated from the IC_{50} values via Eq. 1, using K_m values determined by the oxyhemoglobin assay over a range of arginine and peptide concentrations (murine iNOS: 16 μM).

$$K_i = IC_{50} / (1 + S / K_m) \quad (1)$$

2.5. Western blotting

Western blotting was accomplished according to standard procedures. Total proteins were obtained using a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 lg/mL of leupeptin, 3 mM NaF and 1 mM DTT at 4° C for 30 min. Cell lysates (50 mg of total protein) were resolved on a 4-12% NuPAGE gradient gel (Invitrogen), then electrotransferred onto a nitrocellulose membrane. Nitrocellulose was blocked in Tris-Buffered Saline (TBS)-milk and incubated

overnight with an anti-iNOS primary antibody (1:1000) (Santa Cruz Biotech Inc., Santa Cruz, CA, USA). Nitrocellulose sheets were then washed in TBS, incubated for 1 h with a secondary antibody conjugated with alkaline phosphatase, washed again, and developed in an alkaline buffer with nitroblue-tetrazolium (NBT) as substrate (Alkaline Phosphatase Conjugate Substrate Kit, Bio-Rad, Hercules, CA, USA). β-actin (1/10.000; Sigma, Italy) was used as internal standard. The resulting blot image files were imported and analysed with a commercially available gel-analysis software package (Gel Doc 1000 Bio-Rad, Milan, Italy).

2.6. Semi-quantitative reverse transcription-polymerase chain reaction

RT-PCR was used to determine iNOS mRNA levels in PBAMCs. Total RNA was extracted using 1 mL g^{-1} ULTRASPEC-RNA (Biotech Lab. Inc., Houston, TX, USA), as recommended by the manufacturer. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and quantified spectrophotometrically at 260 nm. First-strand cDNA was generated by adding RNA (1 μg) to a mixture containing 1 mM deoxy-nucleoside-triphosphate (dNTP), 1 U μL^{-1} RNase inhibitor, 2.5 U μL^{-1} Moloney murine leukemia virus reverse transcriptase, 2.5 μM oligo-dT, 5 mM $MgCl_2$, and 10X PCR buffer, to a final volume of 20 μL. Reverse transcription was run at 42 °C for 1 h followed by heat inactivation of reverse transcriptase at 92 °C for 10 min. Ribosomal mRNA (18S) was employed as internal standard and was amplified from the same amount of RNA, to correct for variations among different samples. PCR amplification was performed using a Programmable Thermal Controller (MJ Research Inc., MA, USA). The PCR solution contained 10 μL of first-strand cDNA, 4 μL 10X PCR buffer, 2 mM $MgCl_2$, 2 U mL^{-1} Termophylus aquaticus (Taq) DNA polymerase (Celbio, Milan, Italy), and water, to a final volume of 50 μL. The following primer pairs (MWG-Biotech AG, Italy) were used: sense 5'-CGT AAA GAC CTC TAT GCC AA-3' and antisense 5'-AGC CAT GCC AAA TGT CTC AT-

3’ for iNOS and 18S primers, 0.15 mM of both sense 5’-TAC GGA GCA GCA AAT CCA C-3’ and antisense 5’-GAT CAA AGG ACT GCA GCC TG-3’. Samples were overlaid with mineral oil and subjected to 30 cycles at 95 °C for 60 s, 60 °C for 60 s, and to one cycle at 72 °C for 7 min. PCR products were run on 2% agarose gel electrophoresis and photographed after ethidium bromide staining under UV light. Bands on the gel were scanned using a computerised densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy). Data are expressed as mean±SD intensity of optical density.

2.7. Statistical analyses

Data are given as mean±standard deviation (SD) of at least three separate experiments. In the figures, SD are indicated by vertical bars. Statistical analysis was done by One-Way analysis of variance (ANOVA) with Dunnett's post-hoc test where applicable, using GraphPad Prism version 4 (GraphPad Software, San Diego, USA). ** denotes a p-value < 0.01, * denotes a p-value < 0.05 and p-values greater than 0.05 were deemed not significant.

2.8. Molecular modeling

Molecular models of TP-5 and TPN-2 were built with MOE [27]. The coordinates of the TP-5 peptide were modelled upon those of the RGDVY fragment of the HLA-DQ protein, whose crystallographic structure was retrieved from the Protein Data Bank (PDB ID: 3PL6) [28]. A conformational search on TPN-2 was carried out with the LowModeMD method [29], simulating the solvent implicitly with the GB/SA model. The most stable conformations in a 1-kcal range from the global minimum were rigidly superimposed on the TP-5 template using a best-fit algorithm as implemented in the Open3DALIGN software [30].

3. Results and discussion

Peptides were synthesized via a solid-phase approach, using the standard Fmoc method in a manual reaction vessel (see Materials and Methods for details). Firstly, all products were tested for their ability to inhibit the production of NO from L-arginine by the three NOS isoforms, using an enzymatic assay [25]. Recombinant enzymes from different sources were used, namely murine macrophage iNOS, rat brain nNOS, and bovine eNOS. The inhibitory activity against NOS isoforms was assessed by monitoring the oxidation of oxyhemoglobin to methemoglobin by NO. Table 1 lists the potencies of the products expressed as IC_{50}, namely the concentration able to reduce by 50% the NOS-induced production of NO by L-arginine; selectivity among isoforms is also reported. To confirm that the conversion of oxyhemoglobin to methemoglobin was actually due to NOS activity, the hemoglobin capture assay was also performed as described by Salter and Knowles [26], finding almost identical results (data not shown).

TP-5 is the most active member of the TP series: it inhibits iNOS in the micromolar range (IC_{50} = 15 μM), being a 4-times less potent inhibitor of nNOS (IC_{50} = 60 μM), and a very weak inhibitor of eNOS. The tetrapeptide TP-4 has a low inhibitory potency against both the eNOS and the nNOS isoforms, while it is only half as potent as the lead compound against iNOS (IC_{50} = 30 μM). The shorter peptides TP-2 and TP-3 display very feeble inhibitory activity against all isoforms. All peptides belonging to the N^{ω}-nitro-L-arginine series (TPN) display very poor inhibitory activity against the eNOS isoform, similarly to their TP analogues, but their behaviour versus both iNOS and nNOS differs to some extent. In particular, TPN-5 (IC_{50} = 15 μM) is an inhibitor of iNOS as potent as TP-5, while it displays lower iNOS/nNOS selectivity, since it inhibits nNOS three times as strongly as TP-5. TPN-4 behaves similarly to TP-4. TPN-2 (IC_{50} = 5 μM) and TPN-3 (IC_{50} = 10 μM) were the most active compounds against iNOS: TPN-2 was three times more active than TP-5 and double than TPN-3, whereas their iNOS/nNOS

selectivity was similar. The type of inhibition elicited by TP-5 and TPN-2 was also investigated. Both peptides are competitive inhibitors of iNOS, inhibiting binding of the substrate L-arginine to murine macrophage iNOS with a K_i of 6.7 μM for TP-5 and 2.2 μM for TPN-2. The rate of enzyme activity decreases with increasing concentrations of the two peptides, but it remains constant over time (Fig. 2; only data for TP-5 are shown). Reversible inhibition of murine iNOS enzyme activity was assessed for both peptides, by re-determining iNOS activity after their removal by dialysis. Subsequently, the effect of TP and TPN peptides on PBAMCs derived from blood of healthy donors was evaluated. None of the peptides caused significantly increased cell death versus untreated cells; levels of LDH released following treatment were comparable to those measured in untreated control samples. Also in this case, iNOS inhibitory activity by the peptides was studied via the oxyhemoglobin assay. PBAMCs were treated with peptide (100 μM) for 1 h, then iNOS activity and expression were induced by 24-h incubation with LPS (10 μg mL^{-1}) and IFN-γ (100 U mL^{-1}); enzymatic activities in the absence of peptide, with and without LPS/IFN-γ stimulation, are reported as controls (Fig. 3A).

In the TP series, TP-2, TP-3, and TP-4 displayed similar activities (roughly 50% inhibition of NO production), while TP-5 elicited the highest degree of inhibition (80%). Among TPN analogues, TPN-2 was the most effective (44% inhibition), while TPN-3, TPN-4 and TPN-5 did not decrease iNOS activity. This is surprising in view of the products' ability to inhibit the isolated iNOS isoform, and to reduce iNOS mRNA and iNOS protein expression levels in cells to some extent (see below; Fig. 3B and 3C). Multiple mechanisms control the expression and the activity of NOS isoforms. Although iNOS activity is independent of calcium concentrations, many other specific intracellular regulatory mechanisms might enhance iNOS enzyme activity for TPN-3, TPN-4 and TPN-5. Indeed, one of the post-translational steps converting NOS

polypeptides into the active enzymes is the process of iNOS homodimerization, which in turn is influenced by the availability of its own substrates and co-factors [31]. Studies are in progress in our laboratory to strengthen these preliminary observations and to confirm this hypothesis.

In order to determine whether the modulation of iNOS activity is also related to its expression, the effects of the TP and TPN series on iNOS protein and mRNA levels in LPS/IFN-γ-stimulated cells were examined. Cells were cultured alone, or in the presence of peptide, then pre-incubated at 100 μM 1 h before the 24-h treatment with LPS/IFN-γ. Immunoblotting evidenced a band with an estimated molecular mass of 130 kDa, corresponding to the iNOS protein (Fig. 3B). All the products reduced iNOS protein levels compared to control cells; however, the extent of reduction varied across the series. In the TP series, the effect shows an increasing trend with increasing length of the peptide chain, the most active product being TP-5. This compound reduces iNOS protein expression ($p < 0.01$) by about 45%, while the shortest member of the series TP-2 only reduces it by 15%, with less statistical significance ($p < 0.05$). By contrast, in the TPN series the trend is reversed, the most active product being TPN-2 and the least active TPN-5; these have comparable activity to TP-5 and to TP-2, respectively.

Since the Western blot analyses indicated that the peptides were able to counteract iNOS overexpression induced by LPS/IFN-γ, semi-quantitative reverse-transcribed polymerase chain reaction (RT-PCR) was performed to check whether iNOS mRNA levels were also affected. Total RNA was extracted from PBAMCs and subjected to RT-PCR. The PCR products were separated by agarose gel electrophoresis, and photographed after ethidium bromide staining under UV light. The detected mRNA levels are reported in Fig. 3C. All products, tested at 100 μM, decreased mRNA levels compared to control cells, although with different statistical significances. The trend of the activity in the TP series parallels the one found for iNOS protein

level reduction. Again, the most active product was TP-5 (about 50% reduction of mRNA level, $p < 0.01$), and the least active was TP-2 (about 25% reduction of mRNA level, $p < 0.05$). In the TPN series, the parallelism was looser: the shortest member, TPN-2, was the most effective (about 50% reduction of mRNA level, $p < 0.01$), while the other peptides all had similar activities (about 24% reduction of mRNA level, $p < 0.05$). In summary, TP-5 and TPN-2 exhibited the most potent inhibitory effect on iNOS activity and expression. As shown in Fig. 4, both peptides displayed respectively similar (TPN-2) and significantly greater (TP-5) ability to decrease iNOS activity and expression compared to the positive control L-NAME (100 μM). Finally, the down-regulating effect on iNOS of TP-5 and TPN-2 was investigated in *ex vivo* PBAMCs isolated from two patients affected by SS syndrome, who displayed a significantly higher basal iNOS level compared to healthy subjects (patient 1: 0.46±0.01; patient 2: 0.54±0.01; healthy subjects 0.22±0.02; $p < 0.01$). Following incubation over 24 h at three different concentrations (1, 10, 100 μM), both peptides triggered a reduction of iNOS protein levels, as determined by immunoblotting (Fig. 5A). TP-5 had a significant effect at 10 and 100 μM (at 10 μM, patient 1: 0.22±0.01; patient 2: 0.20±0.06, respectively); TPN-2 was similarly active, but already influenced the iNOS level at a concentration as low as 1 μM (patient 1: 0.23±0.01; patient 2: 0.26±0.02, respectively). Subsequently, we used the oxyhemoglobin assay to assess whether inhibition of iNOS protein expression by the two peptides was accompanied by a parallel change in NO production. Both peptides were able to decrease iNOS activity, TP-5 being slightly more potent (Fig. 5B).

In the attempt to clarify whether a stereoelectronic similarity might underlie the comparable activity profiles of TP-5 and TPN-2, the three-dimensional structure of TP-5 (amino acid sequence RKDVY) was modeled upon the RGDVY motif present in the HLA-DQ protein,

whose crystallographic coordinates are available in the Protein Data Bank. The choice of the template was based on the relationship between thymopentin and the RGDVY fragment established by previous investigators [32]; the resulting conformation is in accordance with evidences gathered from NMR studies [33]. Subsequently, a conformational search on TPN-2 was carried out, and the most stable conformers underwent rigid-body superimposition on the TP-5 scaffold. The best-fitting conformation of TPN-2 shows an excellent match with TP-5, namely between the Lys side chains and the charged terminal amino groups; interestingly, the N^{ω}-nitro moiety of TPN-2 appears to mimic the carboxylate group of the Asp residue in TP-5 (Fig. 6). The synthesis of opportunely constrained analogues is necessary to confirm the role of these pharmacophoric features in the activity of the two peptides.

4. Conclusions

Despite the fact that thymopentin has been used successfully for many years in the experimental treatment of Sézary syndrome, its mechanism of action has remained obscure. In this study we demonstrate that this peptide acts as an inhibitor of the isolated recombinant iNOS and nNOS isoforms, and reduces iNOS protein/mRNA levels and iNOS activity in PBAMCs of both healthy donors and SS patients. Results with TPN-2, the N^{ω}-nitro analogue of the Arg-Lys motif present in TP-5, were similar. The preliminary assays performed so far could not indicate the individual contribution of the two mechanisms of iNOS down-regulation to the overall inhibitory effect in cells. To shed light on this point, additional investigations are necessary, including the assessment of the degree of penetration of these peptides in the cells, and the identification of the intracellular pathways and the transcription factors involved in the reduction of protein

expression. These studies will allow saying a conclusive word about the role of iNOS down-regulation in the therapeutic action of TP-5 against SS.

5. Acknowledgment

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6. Supplementary material

^{1}H-NMR, ^{13}C-NMR and elemental compositions (C, H, N) of the synthesised peptides.

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TP series		TPN series	
TP-2	Arg-Lys	**TPN-2**	$N^{\omega}NO_2$Arg-Lys
TP-3	Arg-Lys-Asp	**TPN-3**	$N^{\omega}NO_2$Arg-Lys-Asp
TP-4	Arg-Lys-Asp-Val	**TPN-4**	$N^{\omega}NO_2$Arg-Lys-Asp-Val
TP-5	Arg-Lys-Asp-Val-Tyr	**TPN-5**	$N^{\omega}NO_2$Arg-Lys-Asp-Val-Tyr

Fig. 1. Sequences of the synthesised peptides.

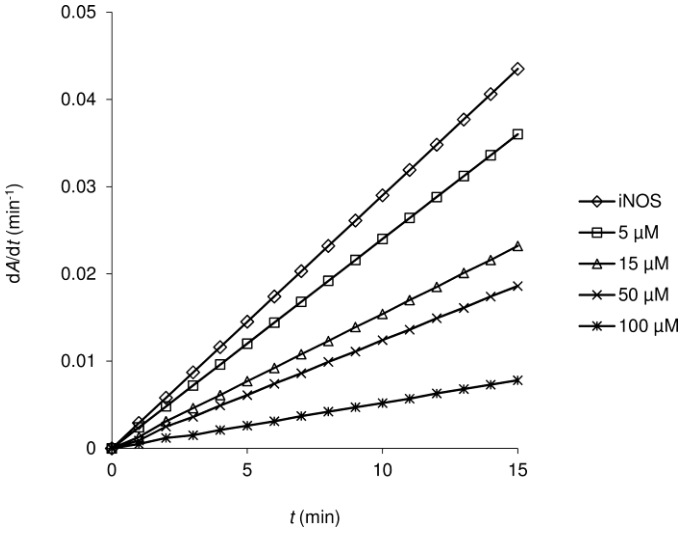

Fig. 2. Time course of murine macrophage iNOS inhibition elicited by TP-5 at increasing concentrations *vs* control (iNOS).

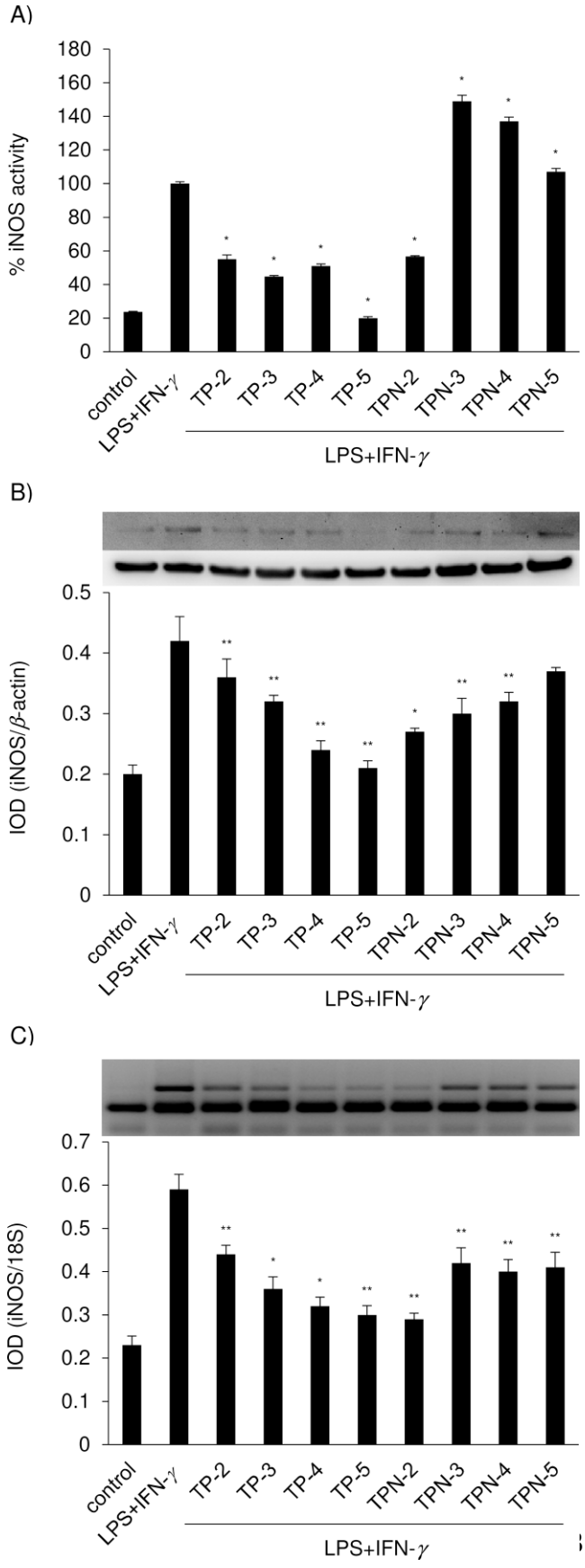
A)
% iNOS activity
180
160
140
120
100
80
60
40
20
0
control
LPS+IFN-γ
TP-2
TP-3
TP-4
TP-5
TPN-2
TPN-3
TPN-4
TPN-5
LPS+IFN-γ
B)
IOD (iNOS/β-actin)
0.5
0.4
0.3
0.2
0.1
0
control
LPS+IFN-γ
TP-2
TP-3
TP-4
TP-5
TPN-2
TPN-3
TPN-4
TPN-5
LPS+IFN-γ
C)
IOD (iNOS/18S)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
control
LPS+IFN-γ
TP-2
TP-3
TP-4
TP-5
TPN-2
TPN-3
TPN-4
TPN-5
LPS+IFN-γ

Fig. 3. A) Percentage of cellular iNOS activity compared to untreated samples (control) and to samples stimulated with LPS/INF-γ, in the absence (LPS+INF-γ) and in the presence of TP/TPN peptides. (** $p < 0.01$, *$p < 0.05$; Dunnett's test *vs* LPS+INF-γ). B) Effects of iNOS protein expression in PBAMCs compared to untreated samples (control) and to samples stimulated with LPS/INF-γ, in the absence (LPS+INF-γ) and in the presence of TP/TPN peptides. The Western blot reported in figure is representative of five independent experiments performed, which all gave similar results. In the densitometric analysis, each bar represents the mean intensity of optical density (IOD)±SD of relative expression of iNOS, normalized to β-actin level (** $p<0.01$, * $p<0.05$; Dunnett's test *vs* LPS+INF-γ). C) RT-PCR analysis of iNOS mRNA expression level in PBAMCs compared to untreated samples (control) and to samples stimulated with LPS/INF-γ, in the absence and in the presence of TP/TPN peptides. The gel shown in figure is representative of three independent experiments performed. In the densitometric analysis, each bar represents the mean IOD±SD of the relative expression of iNOS normalized to 18S level (** $p < 0.01$, * $p < 0.05$; Dunnett's test *vs* LPS+INF-γ).

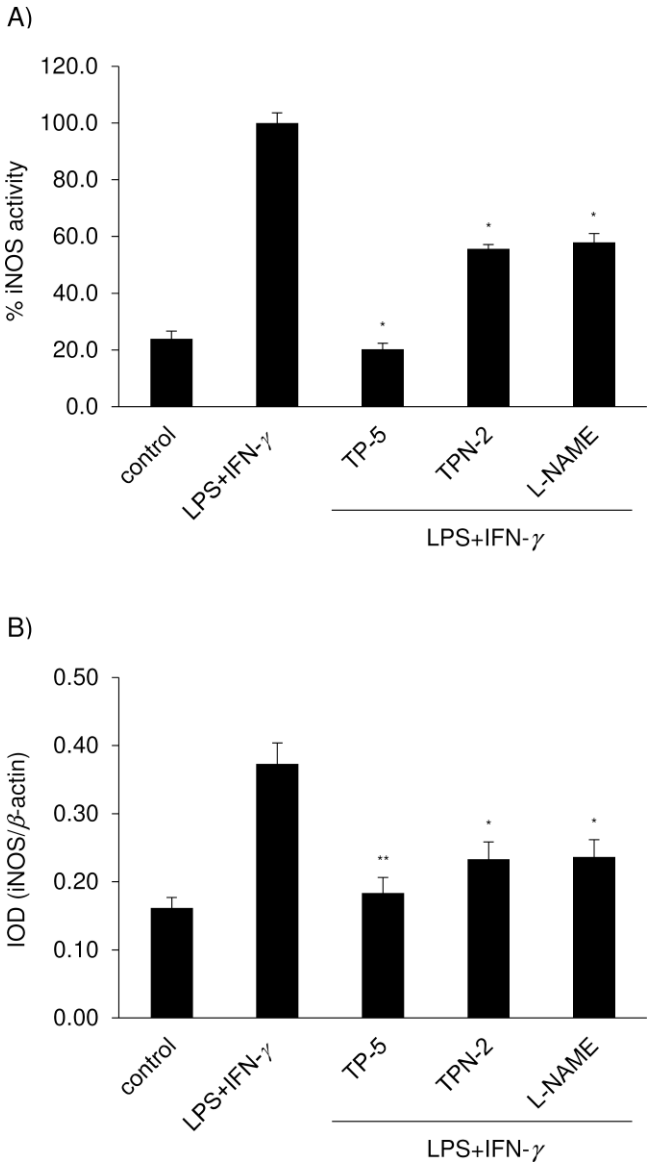

Fig. 4. A) Percentage of cellular iNOS activity compared to untreated samples (control) and to samples stimulated with LPS/INF-γ, in the absence (LPS+INF-γ) and in the presence of TP-5 and TPN-2 peptides. (* $p < 0.05$; Dunnett's test *vs* LPS+INF-γ). B) Effects of iNOS protein expression in PBAMCs compared to untreated samples (control) and to samples stimulated with LPS/INF-γ, in the absence (LPS+INF-γ) and in the presence of TP-5 and TPN-2 peptides. The Western blot reported in figure is representative of three independent experiments performed, which all gave similar results. In the densitometric analysis, each bar represents the mean

IOD±SD of the relative expression of iNOS normalized to β-actin level (** $p < 0.01$, * $p < 0.05$; Dunnett's test *vs* LPS+INF-γ).

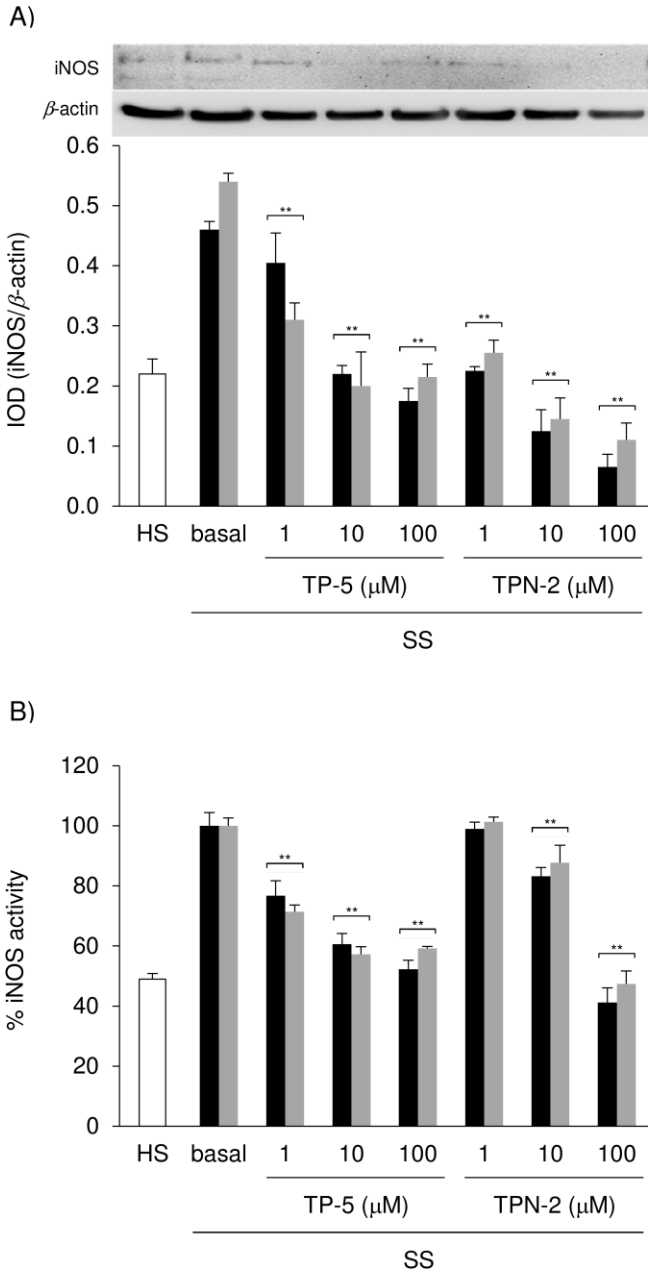

Fig. 5. PBAMCs of two SS patients (black and gray histograms, respectively) were treated *ex vivo* with different concentrations of TP-5 and TPN-2 peptides; healthy subjects (HS, white

histogram) and untreated PBAMCs (basal) are reported as controls. Values are mean±SD of different experiments performed in triplicate (** $p < 0.01$, * $p < 0.05$; Dunnett's test *vs* basal). A) Effects on relative expression of iNOS, normalized to β-actin level. The Western blot reported is representative of three independent experiments performed. In the densitometric analysis, each bar represents the mean IOD±SD of relative expression of iNOS normalized to β-actin level. B) Effects on iNOS activity.

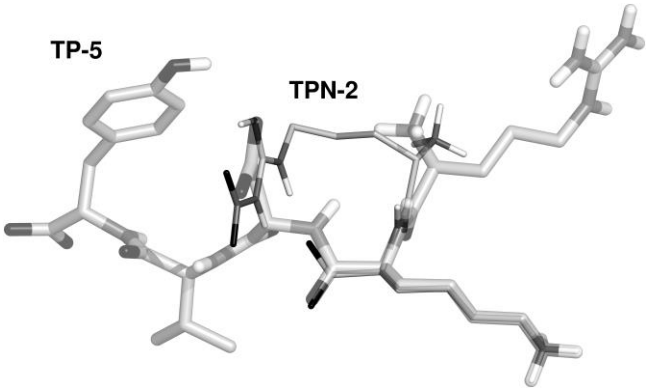

Fig. 6. TP-5 conformation modeled on the RGDVY motif present in the HLA-DQ protein (thicker sticks), superimposed on the best-matching TPN-2 conformation (thinner sticks).